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(71) Applicant: IMMUSOL, INC. [US/US]; 3050 Science Park Road, San Diego, CA 92121 (US).

(72) Inventors: GOLDENBERG, Tsvi; 8727 Cliffridge Avenue, La Jolla, CA 92037 (US). YU, Mang; 7155 B Calabria Court, San Diego, CA 92122 (US). WELCH, Peter, J.; 4689 E. Talmadge Drive, San Diego, CA 92116 (US). BARBER, Jack, R.; 11987 Caneridge Place, San Diego, CA 92128 (US).

(74) Agents: McMASTERS, David, D. et al.; Seed and Berry L.L.P., 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

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(57) Abstract

This invention provides ribozymes useful to treat or prevent Hepatitis B Virus ("HBV") infection in an organism or subject, as well as methods of treating an HBV infection. Reagents such as vectors, host cells, DNA molecules coding for these ribozymes useful in methods of treatment and prevention of HBV infection also are provided.

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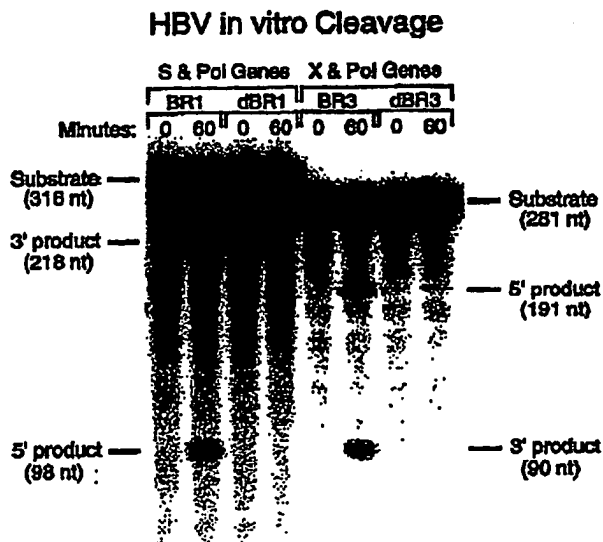
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Description

RIBOZYME THERAPY FOR HEPATITIS B INFECTIONS

Technical Field

The present invention relates generally to therapeutics, and more specifically, to compositions and methods which utilize ribozymes in the treatment of Hepatitis B infections.

Background of the Invention

Hepatitis B viral ("HBV") infection remains a worldwide health problem, with estimates of over 300 million chronically infected individuals. The Center for Disease Control ("CDC") estimates that there are approximately 1 million carriers in the United States and over 300,000 new infections per year. Chronic HBV infection is a major cause of liver cirrhosis. HBV infection also is associated with one of the most common visceral malignancies worldwide, primary hepatocellular carcinoma. In areas of the world where HBV infection is endemic, hepatocellular carcinoma ("HCC") is among the most common lethal malignancies.

Hepatocellular carcinoma is a rapidly fatal tumor unless curative hepatic resection is possible. Unfortunately, most patients with HBV and HCC are cirrhotic and resection is contraindicated. Chemotherapy also has proven to be ineffective. Chemoembolization has shown some recent promise in uncontrolled trials. Orthotopic liver transplantation ("OLT") has been performed for HCC, but the long term survival is less than 25%. Many centers have abandoned orthotopic liver transplantation to treat hepatocellular carcinoma because of the poor outcome and the obligate loss of the donated organ at this time, when there are two suitable recipients for every donor organ.

Alpha 2b interferon has been successfully used to treat chronic HBV infection in adults and is now being studied in children. Prognostic indicators of

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success include a low rate of viral replication and a substantial elevation of serum aminotransferase enzymes. In chronic carriers and patients with modest aminotransferase enzyme elevations, however, treatment is usually unsuccessful. Interferon is also ineffective in immunosuppressed patients with recurrent HBV

5   infection post-OLT. Thus, a need exists for an effective treatment to combat HBV infection. This invention satisfies this need and provides related advantages as well.

Summary of the Invention

The present invention provides ribozymes useful to treat or prevent
10   Hepatitis B Virus ("HBV") infection in an organism or subject, as well as methods of treating an HBV infection. Reagents such as vectors, host cells, DNA molecules coding for these ribozymes useful in methods of treatment and prevention of HBV infection also are provided.

Accordingly, in one aspect the present invention provides ribozymes
15   having the ability to inhibit replication and infectivity of a hepatitis B virus. Preferably, the ribozyme is a hammerhead or hairpin ribozyme, representative examples of which recognize the sequences set forth in Table 1, below. In preferred embodiments, the present invention also provides a nucleic acid molecule encoding such ribozymes; further preferably, the nucleic acid is DNA or cDNA. Even further preferably, the
20   nucleic acid molecule is under the control of a promoter to transcribe the nucleic acid.

In another aspect, the present invention provides host cells containing the ribozymes described herein, vectors comprising the nucleic acid encoding the ribozymes described herein, and host cells comprising such a vector. Preferably, the vector is a plasmid, a virus, retrotransposon, a cosmid or a retrovirus. In one
25   embodiment where the vector is a retroviral vector, the nucleic acid molecule encoding the ribozyme under the control of a promoter, which is preferably a pol III promoter, further preferably a human tRNA^{Val} promoter or an adenovirus VA1 promoter, is inserted between the 5' and 3' long terminal repeat sequences of the retrovirus.

30   The present invention also provides a host cell stably transformed with such a retroviral vector. Preferably, the host cell is a murine or a human cell.

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In a further aspect, the present invention provides methods for producing a ribozyme, the ribozyme being able to inhibit hepatitis B viral infection and replication in a cell, which method comprises providing a nucleic acid molecule (*e.g.*, DNA) encoding the ribozyme under the transcriptional control of a promoter, and

5   transcribing the nucleic acid molecule to produce the ribozyme. Preferably, the method further comprises purifying the ribozyme produced. The ribozyme may be produced *in vitro*, *in vivo* or *ex vivo*.

In yet another aspect, the present invention provides methods of interfering with or preventing HBV replication in a cell infected with HBV, which

10   method comprises introducing into the cell an effective amount of the ribozymes described herein. In one embodiment, such methods comprise introducing into the cell an effective amount of DNA encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme. Preferably, the cell is a human cell.

In still a further aspect, the present invention provides methods of

15   preventing hepatitis B viral infection in a cell susceptible to infection with HBV, which methods comprise introducing into the cell an effective amount of a nucleic acid molecule (*e.g.*, DNA) encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme. Preferably, the cell is a human cell.

In preferred embodiments, the methods further comprise administering

20   the cell transduced with a retroviral vector to a mammal of the same species as that from which the transduced cell was obtained. In other preferred embodiments, the cell transduced with the retroviral vector has been obtained from the mammal receiving the transduced cell.

These and other aspects of the present invention will become evident

25   upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein that describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

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Brief Description of the Drawings

Figure 1 is a schematic illustration of vector pLNT-Rz.

Figures 2A, 2B, 2C and 2D are schematic representations of HBV and ribozyme expression vectors, and wild type and tetraloop hairpin ribozyme structures.

5   Figure 2A discloses "overlength" HBV as cloned into pGem7. The pre-genomic RNA (pgRNA) start site and common polyA signal are indicated. All RNA transcripts are driven by authentic HBV promoters. Cleavage sites for hairpin ribozymes BR1, BR2 and BR3 are indicated by downward arrows. HBV-encoded proteins X, C (core), S (envelope) and P (polymerase) are shown as boxes below. Figure 2B discloses a

10   ribozyme in the retroviral vector pLNT-Rz, which is used for ribozyme expression in mammalian cells. Briefly, ribozymes are cloned under the control of the human tRNA^{val} promoter ("LTR": Moloney Long Terminal Repeat). In Figure 2C, the hairpin ribozyme consists of a 50 to 54 nucleotide RNA molecule (shaded, in uppercase letters) which binds and cleaves an RNA substrate (lowercase letters). The

15   catalytic RNA folds into a 2-dimensional structure that resembles a hairpin, consisting of two helical domains (Helix 3 and 4) and 3 loops (Loop 2, 3 and 4). Two additional helixes, Helix 1 and 2, form between the ribozyme and its substrate. Recognition of the substrate by the ribozyme is via Watson-Crick base pairing (where N or n = any nucleotide, b = C, G or U and B = the nucleotide complementary to b). The length of

20   Helix 2 is fixed at 4 basepairs and the length of Helix 1 typically varies from 6 to 10 basepairs, with 8 being experimentally optimal for most ribozymes. The substrate must contain a GUC in Loop 5 for maximal activity, and cleavage occurs immediately 5' of the G as indicated by an arrow. The catalytic, but not substrate binding, activity of the ribozyme can be disabled by mutating the AAA in Loop 2 to CGU, as shown.

25   The tetraloop hairpin ribozyme (Figure 2D) differs from the hairpin ribozyme by replacement of Loop 3 and Helix 4 with the RNA-stabilizing tetraloop configuration.

Figure 3 depicts the results of *in vitro* cleavage experiments of HBV RNA by BR1 and BR3 ribozymes. Ribozymes BR1 and BR3, as well as their disabled counterparts, dBR1 and dBR3 were tested. Briefly, ribozymes and radiolabeled HBV

30   substrate were synthesized by *in vitro* transcription. HBV substrates are as follows: BR1 substrate = HBV nucleotides 1629-1919 (pgRNA start site is nucleotide 1),

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generated from EcoNI-linearized pGem-HBV-X/B; BR3 substrate = HBV nucleotides 2782-3023, generated from EcoNI-linearized pGem-HBV-B/E. Cleavage reactions contain 40 nM ribozyme and 200 nM substrate (ribozyme:substrate = 1:5) and were incubated for 0 or 60 minutes at 37°C. Cleavage products were resolved on 5%

5 polyarcylamide/7M urea gels. Uncleaved substrate and the 5' and 3' cleavage products are indicated.

Figures 4A and 4B depicts the results of *in vitro* kinetic studies for BR1 and tetraloop BR1 (Figure 4A); and BR3 and tetraloop BR3 (Figure 4B). Shown are typical *in vitro* cleavage reaction timecourse experiments, including 5.2 nM

10 ribozyme and 72 nM substrate. The reactions were incubated at 37°C and aliquots were removed every 30 minutes from 0 to 120 minutes, as indicated. Cleavage products were quantitated by phosphorimager analyses.

Figures 5A and 5B show ribozyme expression *in vitro* verified by RNase protection. Briefly, in Figure 5A Huh7 cells were transfected with each of the

15 ribozymes indicated. Twenty-five micrograms of total cellular RNA was hybridized to 128 nucleotide radiolabeled probes, of equal, specific activity, antisense to either BR1 or BR3, as indicated. Following RNase digestion, reactions were electrophoresed on a 8% polyacrylamide/7M urea gel and quantitated by phosphorimager analysis. Due to the three nucleotide difference between the active and the disabled form of each

20 ribozyme, the expected protected fragments are 64 nt for BR1 and BR3, and 32 nt + 29 nt for dBR1 and dBR3 (see panel 5B for schematic representation of protected fragments).

Figures 6A and 6B show that expression of anti-HBV ribozymes reduces Hepatitis B virus production in liver cell culture. Briefly, Huh7 cells were co-

25 transfected with overlength HBV and pLNT-Rz for each ribozyme. The DNA molar ratio for HBV ribozyme ranged from 1:5 to 1:20, as indicated. Three days post transfection, cells were lysed and HBV particles were immunoprecipitated for the endogenous polymerase assay. Figure 6A shows a typical endogenous polymerase assay with radiolabeled HBV DNA resolved by agarose gel electrophoresis, with

30 relaxed circular (R.C.) and linear forms indicated. Figure 6B shows quantitation as performed by phosphorimager analysis. Values are pixel volumes generated following

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phosphorimager scanning. Percentages listed above graph bars indicate percent reduction of HBV production by each ribozyme, relative to HBV transfections without ribozyme (first bar on graph).

5   Detailed Description of the Invention

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used

10   hereinafter.

"Ribozyme" refers to a nucleic acid molecule which is capable of cleaving a specific nucleic acid sequence. Ribozymes may be composed of RNA, DNA, nucleic acid analogues (e.g., phosphorothioates), or any combination of these (e.g., DNA/RNA chimerics). Within particularly preferred embodiments, a ribozyme

15   should be understood to refer to RNA molecules that contain anti-sense sequences for specific recognition, and an RNA-cleaving enzymatic activity.

"Ribozyme gene" refers to a nucleic acid molecule (e.g., DNA) consisting of the ribozyme sequence which, when transcribed into RNA, will yield the ribozyme.

20   "Vector" refers to an assembly which is capable of expressing a ribozyme of interest. The vector may be composed of either deoxyribonucleic acids ("DNA") or ribonucleic acids ("RNA"). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase, hygromycin

25   phosphotransferase or puromycin-N-acetyl-transferase. Additionally, depending on the host cell chosen and the vector employed, other genetic elements such as an origin of replication, additional nucleic acid restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

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As noted above, the present invention provides ribozymes which are capable of cleaving Hepatitis B viral nucleic acids. Briefly, the viral genome of HBV is about 3000 to 3300 nucleotides in length. Although the viral DNA is circular, both strands of the viral duplex are linear, and the circular conformation is maintained by

5 extensive base-pairing between the two gapped DNA strands. The negative strand of the virus is about 3200 nucleotides in length while the positive strand is shorter, due to single-stranded gaps of variable size. Upon infection, the HBV DNA polymerase in the nucleocapsid core is activated and completes the synthesis of the positive strand, using the negative strand as a template. These features of the genome structure have

10 suggested a retrovirus-like pathway of virus replication.

After conversion of the gapped double stranded DNA to fully double stranded DNA, a full-length positive-strand RNA is transcribed from the DNA template. It is believed that this pregenome RNA is packaged within the cell to form an "immature core," and by reverse transcription, a DNA strand of negative polarity is

15 synthesized. This step is followed by the synthesis of a partial positive strand and the full maturation of the virus particle containing a gapped DNA genome. However, it is the pregenome RNA and the mRNA of the virus which is the target of the ribozymes of this invention. Successful inhibition at this stage in the viral cycle can potentially eliminate hepatic and extrahepatic replication and transmission in all infected patients,

20 including chronic carriers. An additional benefit is the prevention of Hepatitis D viral infection which require HBsAg as its envelope.

RIBOZYMES

As noted above, the present invention provides ribozymes having the

25 ability to inhibit replication, infectivity, or gene expression of a hepatitis B viral infection in a cell. Several different types of ribozymes may be constructed for use within the present invention, including for example, hammerhead ribozymes (Rossi, J.J. et al., *Pharmac. Ther.* *50*:245-254, 1991) (Forster and Symons, *Cell* *48*:211-220, 1987; Haseloff and Gerlach, *Nature* *328*:596-600, 1988; Walbot and Bruening, *Nature*

30 *334*:196, 1988; Haseloff and Gerlach, *Nature* *334*:585, 1988; Haseloff et al., U.S. Patent No. 5,254,678), hairpin ribozymes (Hampel et al., *Nucl. Acids Res.*

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18:299-304, 1990, and U.S. Patent No. 5,254,678), hepatitis delta virus ribozymes (Perrotta and Been, *Biochem. 31*:16, 1992), Group I intron ribozymes (Cech et al., U.S. Patent No. 4,987,071) RNase P ribozymes (Takada et al., *Cell 35*:849, 1983); (*see e.g.*, WO 95/29241, entitled "Ribozymes with Product Ejection by Strand

5   Displacement"; and WO 95/31551, entitled "Novel Enzymatic RNA Molecules."

Cech et al. (U.S. Patent No. 4,987,071, issued January 22, 1991) has disclosed the preparation and use of certain synthetic ribozymes which have endoribonuclease activity. These ribozymes are based on the properties of the *Tetrahymena* ribosomal RNA self-splicing reaction and require an eight base pair

10   target site with a requirement for free guanosine or guanosine derivatives. A temperature optimum of 50°C is reported for the endoribonuclease activity. The fragments that arise from cleavage contain 5'-phosphate and 3'-hydroxyl groups and a free guanosine nucleotide added to the 5'-end of the cleaved RNA. In contrast, the ribozymes of this invention hybridize efficiently to target sequences at physiological

15   temperatures, making them suitable for use *in vivo*, not merely as research tools (*see* column 15, lines 18 to 42, of Cech et al., U.S. Patent No. 4,987,071).

Particularly preferred ribozymes for use within the present invention are hairpin ribozymes (for example, as described by Hampel et al., European Patent Publication No. 0 360 257, published March 26, 1990). Briefly, the sequence

20   requirement for the hairpin ribozyme is any RNA sequence consisting of NNNBN*GUC(N)$_x$ (Sequence ID Nos. 1-5) (where x is any number from 6 to 10, N*G is the cleavage site, B is any of G, C, or U, and N is any of G, U, C, or A). Representative examples of recognition or target sequences for hairpin ribozymes are set forth below in the Examples. Additionally, the backbone or common region of the

25   hairpin ribozyme can be designed using the nucleotide sequence of the native hairpin ribozyme (Hampel et al., *Nucl. Acids Res. 18*:299-304, 1990) or it can be modified to include a "tetraloop" structure that increases stability and catalytic activity (see Example 2 and Figure 3; see also Yu et al., *Virology 206*:381-386, 1995; Cheong et al., *Nature 346*:680-682, 1990; Anderson et al., *Nucl. Acids Res. 22*:1096-1100,

30   1994). The sequence requirement at the cleavage site for the hammerhead ribozyme is any RNA sequence consisting of NUX (where N is any of G, U, C, or A and X

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represents C, U, or A) can be targeted. Accordingly, the same target within the hairpin leader sequence, GUC, is useful for the hammerhead ribozyme. The additional nucleotides of the hammerhead ribozyme or hairpin ribozyme is determined by the target flanking nucleotides and the hammerhead consensus sequence (*see*

5   Ruffner et al., *Biochemistry 29*:10695-10702, 1990).

Given the disclosure provided herein, along with published sequences of the HBV genome and of RNA encoding the surface antigen of HBV, ribozymes of the present invention may be readily constructed. Appropriate base changes in the ribozyme are made to maintain the necessary base pairing with the target HBV

10 sequences. The sequence of the HBV is well known to those of skill in the art and can be obtained from GenBank (*i.e.*, accession numbers V00866, J02201, X00715, for HBV ADW), as well as other sources.

The ribozymes of this invention, as well as DNA encoding such ribozymes and other suitable nucleic acid molecules, described in more detail below,

15 can be chemically synthesized using methods well known in the art for the synthesis of nucleic acid molecules (*see e.g.*, Heidenreich et al., *J. FASEB 70*(1):90-6, 1993; Sproat, *Curr. Opin. Biotechnol. 4*(1):20-28, 1993), . Alternatively, Promega, Madison, Wis., USA, provides a series of protocols suitable for the production of nucleic acid molecules such as ribozymes.

20 Within other aspects of the present invention, ribozymes can also be prepared from a DNA molecule or other nucleic acid molecule (which, upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, *e.g.*, the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention are nucleic acid molecules, *e.g.*, DNA or

25 cDNA, coding for the ribozymes of this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced *in vitro* upon incubation with the RNA polymerase and appropriate nucleotides. In a separate embodiment, the DNA may be inserted into an expression cassette, such as described in Cotten and Birnstiel, *EMBO J. 8*(12):3861-3866, 1989,

30 and in Hempel et al., *Biochemistry 28*:4929-4933, 1989. A more detailed discussion

of molecular biology methodology is disclosed in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989.

During synthesis, the ribozyme can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase (Rossi et al., *Pharmac. Ther. 50*:245-254, 1991). Alternatively, the ribozyme can be modified to the phosphothio analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

VECTORS

Use of ribozymes to treat HBV infection involves introduction of functional ribozyme to the infected cell of interest. This can be accomplished by either synthesizing functional ribozyme *in vitro* prior to delivery, or, by delivery of DNA capable of driving ribozyme synthesis *in vivo*.

More specifically, within other aspects of the invention the ribozyme gene may be constructed within a vector which is suitable for introduction to a host cell (*e.g.*, prokaryotic or eukaryotic cells in culture or in the cells of an organism). Appropriate prokaryotic and eukaryotic cells can be transfected with an appropriate transfer vector containing the nucleic acid molecule encoding a ribozyme of this invention.

To produce the ribozymes with a vector *in vivo*, nucleotide sequences coding for ribozymes are preferably placed under the control of a eukaryotic promoter such as pol III (*e.g.*, tRNA or VA-1 from adenovirus), CMV, SV40 late, or SV40 early promoters. Within certain embodiments, the promoter may be a tissue-specific promoter such as, for example, a liver-specific promoter such as the albumin promoter and the alphafetoprotein promoter (Feuerman et al., *Mol. Cell. Biol. 9*:4204-12, 1989; Camper and Tilghman, *Genes Develop. 3*:537-46, 1989); the alcohol dehydrogenase promoter (Felder, *Proc. Natl. Acad. Sci. USA 86*:5903-07, 1989); the Apolipoprotein B gene promoter (Das et al., *J. Biol. Chem. 263*:11452-8, 1988); the Coagulation protease factor VII gene promoter (Erdmann et al., *J. Biol. Chem. 270*:22988-96, 1995); the Fibrinogen gamma gene promoter (Zhang et al., *J. Biol. Chem. 270*:24287-91, 1995); the Glucokinase gene promoter (Williams et al., *Biochem. Biophys., Res.*

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Comm. 212:272-9, 1995); the Liver phosphofructokinase gene promoter (Levanon et al., *Biochem. Mol. Biol. Int.* 35:729-36, 1995); the Phospho-Enol-Pyruvate Carboxy-Kinase ("PEPCK") promoter (Hatzogiou et al., *J. Biol. Chem.* 263: 17798-808, 1988; Benvenisty et al., *Proc. Natl. Acad. Sci. USA 86*:1118-22, 1989; Vaulont et al.,

5   *Mol. Cell. Biol. 9*:4409-15, 1989); or lymphoid-specific promoters. Ribozymes may thus be produced directly from the transfer vector *in vivo*.

A wide variety of vectors may be utilized within the context of the present invention, including for example, plasmids, viruses, retrotransposons and cosmids. Because HBV infects the liver, vectors with hepatotrophic properties are

10   particularly preferred. Representative examples include adenoviral vectors (*e.g.*, WO 94/26914, WO 93/9191; Yei et al., *Gene Therapy 1*:192-200, 1994; Kolls et al., *PNAS 91*(1):215-219, 1994; Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Guzman et al., *Circulation 88*(6):2838-48, 1993; Guzman et al., *Cir. Res.* 73(6):1202-1207, 1993; Zabner et al., *Cell 75*(2):207-216, 1993; Li et al., *Hum Gene Ther.* 4(4):403-

15   409, 1993; Caillaud et al., *Eur. J. Neurosci.* 5(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (*see* WO 95/13365; Flotte et al., *PNAS 90*(22):10613-10617, 1993), hepatitis delta vectors, live, attenuated delta viruses and herpes viral vectors (*e.g.*, U.S. Patent No. 5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320.

20   Other representative vectors include retroviral vectors (*e.g.*, EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). For vectors without inherent hepatotropism (*e.g.*, AAV or retroviruses), cell tropism can be altered to specifically target these viruses to the liver. Methods of using such vectors in gene therapy are

25   well known in the art, see, for example, Larrick, J.W. and Burck, K.L., *Gene Therapy: Application of Molecular Biology*, Elsevier Science Publishing Co., Inc., New York, New York, 1991 and Kreigler, M., *Gene Transfer and Expression: A Laboratory Manual*, W.H. Freeman and Company, New York, 1990.

Further provided by this invention are vectors having more than one

30   nucleic acid molecule encoding a ribozyme of this invention, each molecule under the control of a separate eukaryotic promoter or alternatively, under the control of single

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eukaryotic promoter. Representative examples of other therapeutic molecules which may be delivered by the vectors of the present invention include interferon (*e.g.*, alpha, beta or gamma), as well as a wide variety of other cytokines or growth factors. These vectors provide the advantage of providing multi-functional therapy against HBV 5 infection, preferably with the various therapies working together in synergy.

Host prokaryotic and eukaryotic cells stably harboring the vectors described above also are provided by this invention. Suitable host cells include bacterial cells, rat cells, mouse cells, and human cells, for example, liver and blood cells.
10

DELIVERY

Within certain aspects of the invention, ribozyme molecules, or nucleic acid molecules which encode the ribozyme, may be introduced into a host cell utilizing 15 a vehicle, or by various physical methods. Representative examples of such methods include transformation using calcium phosphate precipitation (Dubensky et al., *PNAS* *81*:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., *Nature 352*:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in 20 order to transiently polarize the membrane, allowing entry of the nucleic acid molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS 89*:6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), polycation compounds such as 25 polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther. 29*:69, 1985; and Friedmann et al., *Science 244*:1275, 1989), and DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 30 1989). In one embodiment, the ribozyme is introduced into the host cell using a liposome.

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PHARMACEUTICAL COMPOSITIONS

As noted above, pharmaceutical compositions also are provided by this
5   invention. These compositions contain any of the above described ribozymes, DNA
molecules, vectors or host cells, along with a pharmaceutically or physiologically
acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic
to recipients at the dosages and concentrations employed. Ordinarily, the preparation
of such compositions entails combining the therapeutic agent with buffers,
10   antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues)
polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or
dextrins, chelating agents such as EDTA, glutathione and other stabilizers and
excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are
exemplary appropriate diluents.

15   In addition, the pharmaceutical compositions of the present invention
may be prepared for administration by a variety of different routes, including for
example intraarticularly, intracranially, intradermally, intrahepatically, intramuscularly,
intraocularly, intraperitoneally, intrathecally, intravenously (e.g., into the portal vein),
or subcutaneously. In addition, pharmaceutical compositions of the present invention
20   may be placed within containers, along with packaging material which provides
instructions regarding the use of such pharmaceutical compositions. Generally, such
instructions will include a tangible expression describing the reagent concentration, as
well as within certain embodiments, relative amounts of excipient ingredients or
diluents (e.g., water, saline or PBS) which may be necessary to reconstitute the
25   pharmaceutical composition

THERAPEUTIC METHODS

Methods of interfering with or preventing HBV viral replication,
30   infectivity, or gene expression in a cell infected with HBV are also provided by this
invention. Such methods require contacting the cell with an effective amount of

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ribozyme of this invention or, alternatively, by transducing the cell with an effective amount of vector having a nucleic acid molecule encoding the ribozyme. Effective amounts are easily determined by those of skill in the art using well known methodology. When exogenously delivering the ribozyme, the RNA molecule can be

5   embedded within a stable RNA molecule or in another form of protective environment, such as a liposome. Alternatively, the RNA can be embedded within RNase-resistant DNA counterparts. Cellular uptake of the exogenous ribozyme can be enhanced by attaching chemical groups to the DNA ends, such as cholesteryl moieties (Letsinger et al., *P.N.A.S., U.S.A.*, 1989).

10   In another aspect of the invention, the target cell is transduced under conditions favoring insertion of the vector into the target cell and stable expression of the nucleic acid encoding the HBV specific ribozyme. The target cell can include but is not limited to hepatocytes and lymphocytes. If the cell is transduced prior to HBV infection, infection of the target cell or its progeny can be prevented. Accordingly,

15   this aspect includes methods for interfering with or preventing HBV viral infection and/or replication in a cell by reacting the target RNA sequence with a ribozyme of this invention.

            In one embodiment of this aspect of the invention, appropriate host cells such as liver cells are removed from a subject, *e.g.*, a human patient, using

20   methods well known in the art. The cells are then trypsinized and resuspended for *ex vivo* therapy. Within the cell or within the cells of an organism, a transfer vector as described above encoding one or more ribozymes is transfected into a cell or cells using methods described in Llewellyn et al., *J. Mol. Biol. 195*:115-123, 1987, and Hanahan, *166*:557-580, 1983. Inside the cell, the transfer vector replicates and the

25   DNA coding for the ribozyme is transcribed by cellular polymerases to produce ribozymes which then inactivate HBV. Micromanipulation techniques such as microinjection also can be used to insert the vector into the cell so that the transfer vector or a part thereof is integrated into the genome of the cell. Transcription of the integrated material gives rise to ribozymes which then inactivate HBV. The above

30   methods are not intended to limit the invention, but merely to exemplify various means

15

to effect the ribozyme therapy of this invention.   Other methods are detailed in Anderson, *Science 256*:808-813, 1992.

      For *ex vivo* therapy, the transduced cells can be reintroduced into the patient by hepatic artery injection under conditions such that the transduced cells will

5   integrate into the liver.

      As used herein, the term "interfering with or preventing" HBV viral replication in a cell means to reduce HBV replication or production of HBV components necessary for progeny virus in a cell as compared to a cell not being transiently or stably transduced with the ribozyme or a vector encoding the ribozyme.

10   Simple and convenient assays to determine if HBV viral replication has been reduced include an ELISA assay for the presence, absence, or reduced presence of anti-HBV antibodies or hepatitis B antigens in the blood of the subject, RT-PCR or liver function tests. Such methods are well known to those of ordinary skill in the art. Alternatively, total RNA from transduced and infected "control" cells can be isolated and subjected

15   to analysis by dot blot or northern blot and probed with HBV specific DNA to determine if HBV replication is reduced.   Alternatively, reduction of HBV protein expression can also be used as an indicator of inhibition of HBV replication. A greater than fifty percent reduction in HBV replication as compared to control cells typically quantitates a prevention of HBV replication.

20

      The following examples are offered by way of illustration, and not by way of limitation.

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EXAMPLES

EXAMPLE 1

CRITERIA FOR HAIRPIN RIBOZYME SITE SELECTION

5

Hairpin ribozymes suitable for use within the present invention preferably recognize the following sequence of HBV RNA: NNNBN*GUC(N)$_x$ (Sequence ID No. 1) (where x is any number from 6 to 10, N*G is the cleavage site, B is any of G, C, or U, and N is any of G, U, C, or A). The sequence GUC must be

10 conserved for all hairpin ribozymes described below. Other nucleotides ("N" as provided above) preferably have a high degree of sequence conservation in order to limit the need for multiple ribozymes against the same target site. GUC hairpin ribozyme recognition sites for HBV are provided below in Table 1.

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Table 1

Hairpin Ribozyme Recognition Sites in Human Hepatitis B
HBV adw subtype (Numbered from unique EcoRI site)

5   NUCLEOTIDE POSITION

	SEQUENCE (5' to 3')	NAME	Seq. ID No.
311	UCGCA^GUCCCCAACCU	BR1	2
343	CUCCU^GUCCUCCAAUU		3
651	CCUCA^GUCCGUUUCUC		4
1303	AGCCG^GUCUGGAGCAA		5
1337	AUUCU^GUCGUCCUCUC		6
1429	GUCCC^GUCGGCGCUGA	BR2 (no cleavage)	7
1494	UCUCC^GUCUGCCGUUC		8
1538	ACGCG^GUCUCCCCGUC		9
1546	UCCCC^GUCUGUGCCUU	BR3	10
1568	UGCCG^GUCCGUGUGCA		11
3127	CGGCA^GUCAGGAAGGC		12
3171	AGACA^GUCAUCCUCAG		13
CONSENSUS	NNNCN^GUCNNNNNNNN	(12 total)	

	SEQUENCE (5' to 3')		Seq. ID No.
113	AUCUC^GUCAAUCUCCG		14
355	AAUUU^GUCCUGGUUAU		15
469	CGUUU^GUCCUCUAAUU		16
611	CCAUC^GUCCUGGGCUU		17
811	CUUUU^GUCUCUGGGUA		18
1151	CUGUC^GUCCUCUCGCG		19
1164	GCCUG^GUCUGUGCCAA		20
1482	CUCUC^GUCCCCUUCUC		21
1792	AAUUG^GUCUGCGCACC		22
2151	UAGUA^GUCAAUUAUGU		23
2253	AUUUG^GUCUCUUUCGG		24
CONSENSUS	NNNUN^GUCNNNNNNNN	(11 total)	

	SEQUENCE (5' to 3')		Seq. ID No.
33	CCAGA^GUCAGGGGUCU		25
40	CAGGG^GUCUGUAUCUU		26
241	GCAGA^GUCUAGACUCG		27
292	CGUGU^GUCUUGGCCAA		28
301	CGUGA^GUCCCUUUAUA		29
376	GAUGU^GUCUGCGGCGU		30
999	UGUGG^GUCUUUUGGGC		31
2024	UUAGA^GUCUCCUGAGC		32
2351	UGCGG^GUCACCAUAUU		33
2416	GCCGC^GUCGCAGAAGA		34
2582	UAGGU^GUCAACAAUUU		35
2951	ACAGU^GUCAACAAUUC		36
CONSENSUS	NNNGN^GUCNNNNNNNN	(12 total)	

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EXAMPLE 2

CONSTRUCTION OF HAIRPIN RIBOZYMES

5   Two single-stranded DNA oligonucleotides are chemically synthesized
such that, when combined and converted into double-stranded DNA, they contain the
entire hairpin ribozyme, including nucleotides complementary to the target site. In
addition, restriction enzyme recognition sites may be placed on either end to facilitate
subsequent cloning.

10   For example, in order to construct ribozyme BR3:

5'-GCGGATCCAAGGCACAAGAAGCGAACCAGAGAAACACACG-3'

                         ||||||||||||||||||

                  3'-TGGTCTCTTTGTGTGCAACACCATATAATGGACCATGCGCAGGG-5'
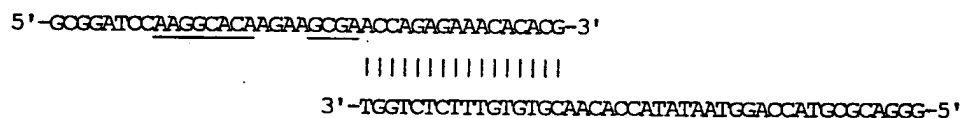
15

          Restriction enzyme sites are in bold. Sequences complementary to the
BR3 target site in HBV are underlined.

          Briefly, the indicated oligonucleotides are hybridized together and
converted to double-stranded DNA using either Klenow DNA polymerase or Taq

20   DNA polymerase. The resulting DNA is cleaved with restriction enzymes *Bam*HI and
*Mlu*I, purified and cloned into vectors for *in vitro* transcription (pGEM, ProMega,
Madison, Wis.) or for retrovirus production and mammalian expression (pLNL/MJT
backbone).

          Defective or "disabled" ribozymes for use as controls may be

25   constructed as described above, with the exception that the sequence AAA is changed
to a UGC as shown in Figure 2.

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EXAMPLE 3

IN VITRO CLEAVAGE ASSAYS

Ribozymes are cloned into *in vitro* transcription vectors (pGEM-7Z,
5   ProMega, Madison, Wis.) and transcribed *in vitro* by T7 RNA polymerase. Following
transcription, reactions are treated with RQ1 DNase (Promega, Madison, Wisconsin)
according to the manufacturer's instructions, and the ribozymes are purified by
denaturing polyacrylamide gel electrophoresis.

The full length HBV genome, in pEco63 was obtained from American
10 Type Culture Collection (ATCC Accession Numbers V00866, J02201 and X00715).
Plasmids for HBV substrate production were constructed by cloning two HBV
fragments into pGem7Z as follows. pGem-HBV-X/B was created by ligating the
*Xba*I/*Bam*HI fragment of HBV (nucleotides 1629-2782, pgRNA start site = 1) into
pGem7Z digested with *Xba*I and *Bam*HI. pGem-HBV-B/E was created by ligating
15 the *Bam*HI/*Eco*RI fragment of HBV (nucleotides 2782-1380, pgRNA start site = 1)
into pGem7Z digested with *Bam*HI and *Eco*RI. Both plasmids were linearized with
*Eco*NI prior to *in vitro* transcription. HBV substrates were then synthesized in the
presence of alpha-^{32}P[UTP], DNase treated and gel purified, as described by the
manufacturer (Promega). The *in vitro* cleavage reactions contained 40 nM ribozyme
20 and 200 nM substrate in 12 mM $MgCl_2$/2 mM spermidine/40 mM Tris-HCl, pH 7.5,
were incubated at 37°C for 60 minutes and were terminated by the addition of 7M
urea gel loading buffer. Products of the cleavage reactions were resolved by
electrophoresis on 5% polyacrylamide/7 M urea gels and analyzed by
autoradiography.
25   Results are shown in Figure 3. Briefly, ribozymes BR1 and BR3
cleaved up to 35% of their substrate RNAs in 60 minutes, under physiological
concentrations of magnesium, without any prior treatment or denaturation. As
controls, the disabled forms of BR1 and BR3 (designated dBR1 and dBR3) were also
tested. As described above in Example 2, disabled ribozymes are made by introducing
30 a three nucleotide mutation in loop 2 of the hairpin ribozyme. This inactivates its
RNA cleavage activity, without affecting its substrate binding. Neither dBR1 nor

20

dBR3 showed any RNA cleavage activity (lanes 4 and 8), indicating that the cleavage demonstrated by BR1 and BR3 is specific. Ribozyme BR2, however, showed no *in vitro* cleavage activity after 60 minutes (data not shown). Since both the BR2 ribozyme and its substrate target site were verified by DNA sequencing, the lack of

5   cleavage activity was presumably due to RNA secondary structure within the substrate.

EXAMPLE 4

10   · RIBOZYME KINETIC STUDIES

A.   Preparation of ribozymes and substrates

Ribozyme and substrate synthesis was achieved by plasmid independent *in vitro* transcription. Briefly, oligonucleotides were synthesized (Retrogen, San

15   Diego) that contained the T7 RNA polymerase promoter and the ribozyme or substrate sequences. The universal T7 promoter oligo contains 63 nucleotides of random sequence ("template arm") and 17 nucleotides that make up the T7 promoter. The ribozyme oligo contains the 17 nucleotides complementary to the T7 promoter followed by a CCC (for optimal transcription) and the 50 bases encoding the

20   ribozyme. Similarly, the substrate oligos contain the T7 promoter, a CCC and the 33 or 34 nucleotides encoding the substrate. To synthesize each ribozyme or substrate the two oligonucleotides were annealed and converted to double-stranded DNA using Klenow polymerase.

In vitro transcription was carried using 4 µg of dsDNA template at

25   37°C for 2 hours using T7 RNA polymerase (Promega), according to the manufacturer's instructions. RNAs were labeled by incorporation of alpha P-CTP during the transcription reaction and purified by denaturing gel electrophoresis.

(b)   *In Vitro* Cleavage Reactions.

30   Time course reactions were prepared in a final volume of 25 µl with a ribozyme concentration of 0.0052 µM and substrate concentration of 0.072 µM. The

21

reactions were assembled on ice and 5 μl was removed for the 0 min time point and
mixed with 5 μl loading buffer and stored at -85°C. The remainder of the reaction
was incubated at 37°C for up to 2 hr in 40 mM Tris pII 7.5, 10 mM MgCl₂ mM
spermidine, with timepoints being taken every 30 minutes. Five microliters of reaction

5   was removed for each time point and mixed with loading buffer as above. Reaction
products were analyzed by urea denaturing polyacrylamide gel electrophoresis
(PAGE) and cleavage products were quantitated by phosphorimager analysis
(Molecular Dynamics). Percent cleavage was defined as (radioactivity of the
products)/[(radioactivity of the products) + (radioactivity of remaining substrate)].

10   The Michaelis constant (K_m) and k_{cat} were also determined for each of
the ribozymes by performing multiple turnover kinetic experiments. Briefly, kinetic
reactions were assembled on ice in a final volume of 10 μl. Ribozyme concentration
was held constant at either 2 or 4 nM and substrate concentrations ranged from 2 to
200 nM. Reactions were incubated at 37°C for as little as 15 minutes for the fastest

15   reactions and up to an hour for the slowest reactions. Cleavage reactions were done
in the same buffer as described above and reactions were terminated by the addition of
10 μl of loading buffer. Products were resolved by urea denaturing PAGE and
analyzed as above. The K_m and k_{cat} for the ribozymes were estimated from plotting
the data in an Eadie Hofstee plot (Deltagraph, Deltapoint Inc.) with R^2 >0.90. The

20   error repeated experiments <20%. Catalytic efficiency = k_{cat}/K_m = 10^4 min⁻¹ M⁻¹.

            Results are shown in Figure 4. Briefly, by performing cleavage
timecourse experiments using each ribozyme, it was revealed that in both cases the
addition of a tetraloop enhanced cleavage activity. To determine if the tetraloop
affected K_m, k_{cat} or both, kinetic studies were performed by holding the ribozyme

25   concentration constant while varying the amount of substrate. By plotting this
information in an Eadie-Hofstee plot, K_m and k_{cat} can be determined, and the values for
each ribozyme are shown under the graphs. For BR1 and BR3, the tetraloop
increased the catalytic efficiency 3- to 7.5-fold, respectively, over that of the
unmodified ribozyme. In both cases, addition of a tetraloop mainly enhanced k_{cat},

30   while having only a modest to insignificant impact on K_m.

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EXAMPLE 5

CONSTRUCTION AND TESTING OF HBV RIBOZYME MAMMALIAN EXPRESSION

VECTORS

5

A.   Construction of Vectors

Plasmid pMJT (Yu et al., *Proc. Nat'l Acad. Sci. USA 90*:6340-6344,

1993), which contains the anti-U5 HIV ribozyme driven by the tRNA^{val} RNA pol III

10   promoter, is digested with *Bam*HI and *Mlu*I, and the vector purified from the

ribozyme fragment.

The "overlength" HBV plasmid was constructed by ligating two HBV

DNA fragments into pGEM7Z (Promega). The 5' end of HBV was supplied by the

1966 bp *Sph*I/*Eco*R1 HBV fragment (nucleotides 2614-1380, pgRNA start site = 1),

15   and the 3' portion of HBV consisted of the 1988 bp *Eco*R1/*Bgl*II HBV fragment

(nucleotides 1380-166). A three-way directional ligation was performed wherein the

two fragments were ligated into pG7Z digested with *Sph*I and *Bam* HI). The resulting

plasmid, pGEM-HBV-overlength, contains the entire pgRNA and polyA signal, as

well as the authentic HBV pgRNA promoter. When transfected into human liver cells,

20   this plasmid will express HBV RNA and proteins, resulting in the secretion of

infectious hepatitis B viral particles.

B.   Cell culture, transfection and ribozyme expression.

Huh7 cells were cultured at 37°C in DMEM (Gibco BRL)

25   supplemented with 10% FBS, L-glutamine, sodium pyruvate and antibiotics. For

DNA transfections, 1 x 10^6 cells on 100 mm plates were transfected with 1 μg pGEM-

HBV-overlength plus 5 to 20 μg pLNT-Rz by standard calcium phosphate

transfection procedures, using empty pLNT vector (containing no Rz sequences) to

bring the total DNA to 20 μg. One to three days following transfection, ribozyme

30   expression was verified by RNase protection, according to the manufacturer

(Promega). Results of this experiment are shown in Figures 5A and 5B.

23

C.   Endogenous polymerase assay

Quantification of HBV virus particles produced in tissue culture is best achieved using the endogenous polymerase assay (EPA). This assay relies on the fact

5   that the packaged HBV genome is only partially double-stranded and that the HBV-specific polymerase is also packaged in each virion. Addition of radiolabeled nucleotide triphosphates to purified HBV particles allows the endogenous polymerase to "fill-in" the partially single-stranded region, thus allowing detection of HBV replication and virus production.

10   Briefly, Huh7 cells, co-transfected with pGEM-HBV-overlength and pLNT-Rz DNA, were lysed with EPA Lysis Buffer (50 mM Tris, pH 7.5, 40 mM NH_4Cl, 15 mM $MgCl_2$, 1% NP-40, 0.3% β-mercaptoethanol) for 30 minutes on ice. Lysates were centrifuged at 14,000 x g for 10 minutes at 4°C and HBV core particles in the supernatants were subjected to immunoprecipitation with anti-HBcAg

15   polyclonal antibodies (Dako) on proteinA-sepharose beads (Pharmacia). Immunoprecipitates were washed twice with EPA Lysis Buffer and incubated with 50 µCi alpha-$dCTP^{32}$ + 100 µM each dATP, dGTP and dTTP at 37°C for 2 hours, with occasional mixing. 100 µM cold dCTP was added and the incubation continued for another 1 hour. HBV DNA was purified from the intact particles by digestion with

20   Proteinase K (500 µg/ml final) and SDS (1% final) for 2 hours at 37°C followed by phenol extraction and ethanol precipitation. Purified DNA was separated on 0.8% agarose gels, dried and analyzed by autoradiography and phosphorimager analysis (Molecular Dynamics).

25   D.   Results

The effectiveness of anti-HBV ribozymes as therapeutic agents *in vivo* depends on their ability to function in a complex cellular environment. Briefly, in order to evaluate the ability of each anti-HBV ribozyme to inhibit HBV replication and production, each pLNT-Rz was co-transfected with the "overlength" HBV into Huh7

30   cells and virus production was monitored by EPA. In a typical cotransfection experiment, HBV particles subjected to EPA produce DNA bands representing the

24

fully-repaired relaxed circular (R.C.) form as well as faster migrating linear forms (Figure 6a). When overlength HBV was co-transfected with BR1, HBV production was reduced by up to 77% (Figure 6). Cotransfection with dBR1, however, had no inhibitory effect on HBV replication (lane 6). The lack of an effect by dBR1 strongly

5   suggest that the effects of BR1 are due to *in vivo* RNA cleavage and not simply an "antisense" effect, since dBR1 maintains a perfect complementary target sequence, but is catalytically inactive. Furthermore, co-transfection with increasing molar amounts of BR1, from 1:5 to 1:20 (lanes 5, 4 and 3) resulted in an increasing anti-HBV effect (Figure 6B) indicating a dose-response to the presence of more ribozyme *in vivo*.

10   Cotransfection of BR3 with HBV resulted in an 11% reduction in HBV replication (Fig. 6B). While the observed difference between BR1 and BR3 activities *in vivo* correlates with their *in vitro* cleavage activities, the reduced effectiveness of BR3 *in vivo* may further indicate RNA secondary structure in the full length HBV RNAs or the presence of RNA binding proteins at or around the BR3 target site *in vivo*.

15   Together, these data indicate that both BR1 and BR3 HBV ribozymes can function within human cells and further suggest that expression of anti-HBV ribozymes could potentially inhibit HBV replication in infected livers.

20   From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

25

Claims

We claim: .

1.   A hairpin ribozyme having the ability to inhibit replication and infectivity of a hepatitis B virus.

2.   A nucleic acid molecule encoding the ribozyme of claim 1.

3.   The nucleic acid molecule of claim 2 wherein the nucleic acid is DNA or cDNA.

4.   The nucleic acid molecule of claim 2 under the control of a promoter to transcribe the nucleic acid.

5.   A host cell containing the ribozyme of claim 1.

6.   A vector comprising the nucleic acid of claim 4.

7.   A host cell comprising the vector of claim 6.

8.   The vector of claim 6 wherein the vector is a plasmid, a virus, retrotransposon or a cosmid.

9.   The vector of claim 8 wherein the virus is a retrovirus.

10.   The retroviral vector of claim 9 wherein the retrovirus has inserted between the 5' and 3' long terminal repeat sequences of the retrovirus a nucleic acid molecule according to claim 2 under the control of a promoter.

11.   The retroviral vector of claim 8 wherein the promoter is a pol III promoter.

12.   The retroviral vector of claim 11 wherein the pol III promoter is the human tRNA^{Val} promoter or the adenovirus VA1 promoter.

13.   A host cell stably transformed with the retroviral vector of claim 9.

14.   The host cell of claim 13 wherein the host cell is a murine or a human cell.

15.   A method for producing a ribozyme, the ribozyme being able to inhibit hepatitis B viral infection and replication in a cell, which comprises providing DNA encoding the ribozyme under the transcriptional control of a promoter, transcribing the DNA to produce the ribozyme.

16.   The method of claim 15, further comprising purifying the ribozyme produced.

17.   The method of claim 15 wherein the ribozyme is produced *in vitro*.

18.   The method of claim 15 wherein the ribozyme is produced *in vivo*.

19.   A method of interfering with or preventing hepatitis B virus (HBV) replication in a cell infected with HBV, which comprises introducing into the cell an effective amount of the ribozyme of claim 1.

20.   A method of interfering with or preventing hepatitis B virus (HBV) replication in a cell infected with HBV, which comprises introducing into the cell an effective amount of DNA of claim 4 and transcribing the DNA to produce the ribozyme.

21.   The method of claim 19 or 20 wherein the cell is a human cell.

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22.   A method of preventing hepatitis B viral infection in a cell susceptible
to infection with HBV, which comprises introducing into the cell an effective amount of the
DNA of claim 4 and transcribing the DNA to produce the ribozyme.

23.   The method of claim 22 wherein the cell is a human cell.

24.   The method of claim 22 further comprising administering the cell
transduced with the retroviral vector to a mammal of the same species as that from which the
transduced cell was obtained.

25.   The method of claim 24 wherein the cell transduced with the retroviral
vector has been obtained from the mammal receiving the transduced cell.

FIGURE 1

FIGURE 2

FIGURE 3

HBV in vitro Cleavage
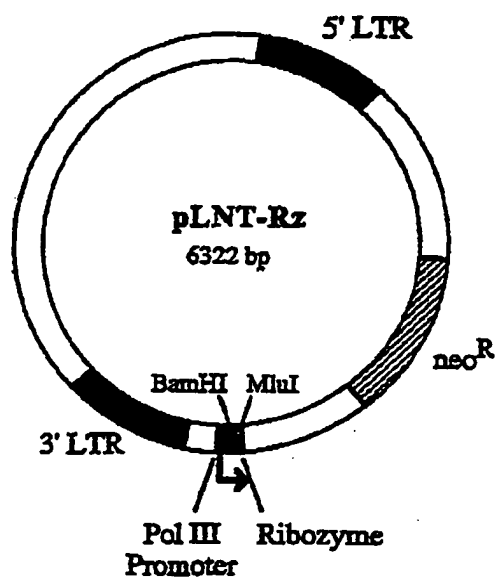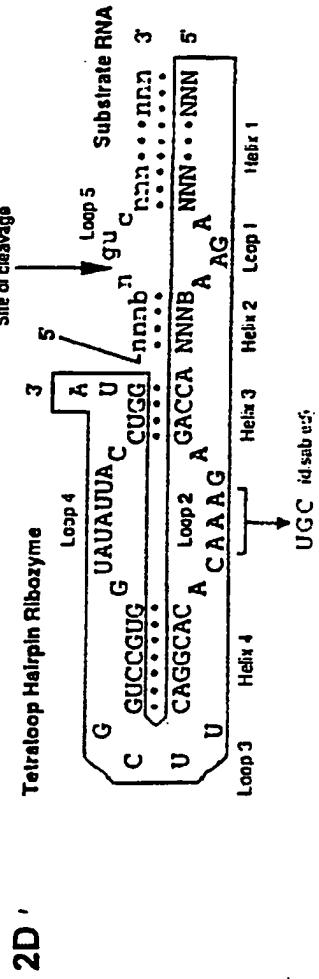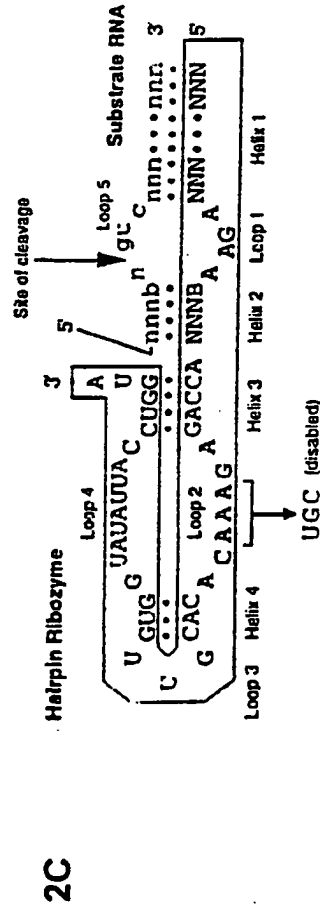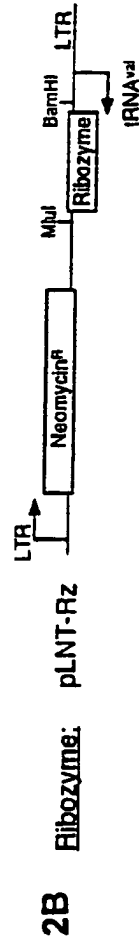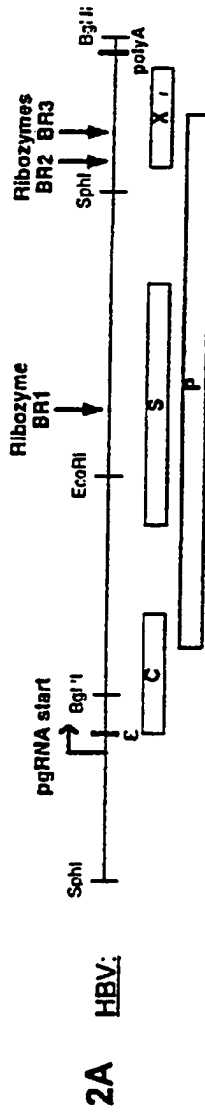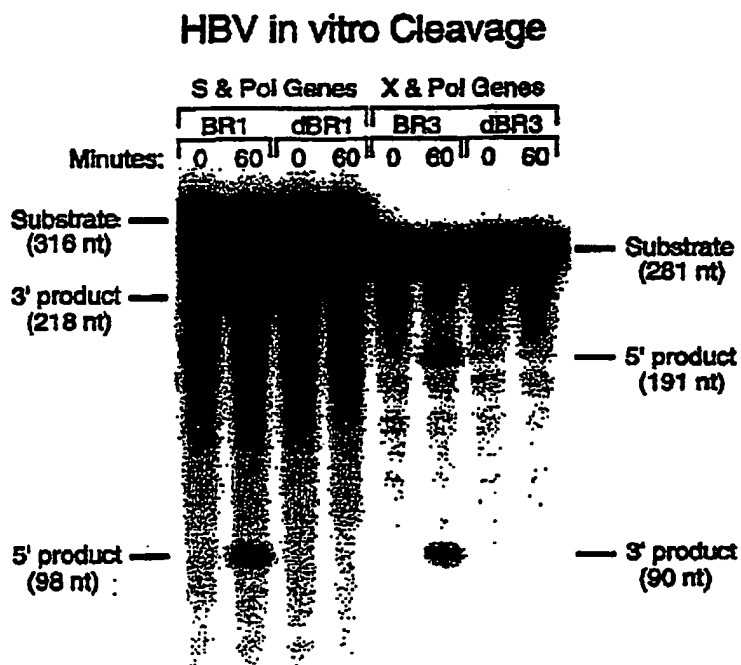
FIGURE 4

4A
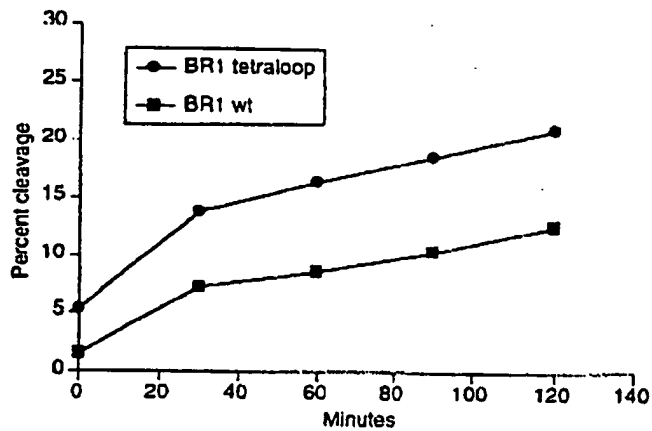
	Km	kcat	kcat/Km
BR1 wt	8.4 nM	0.0560/min	667
BR1 tetraloop	8.7 nM	0.1700/min	1983

4B
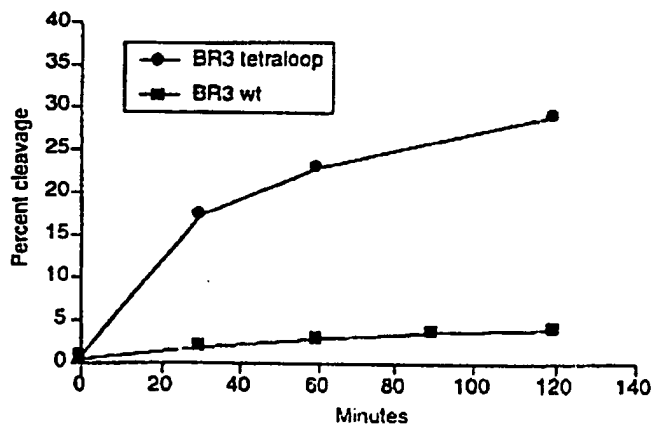
	Km	kcat	kcat/Km
BR3 wt	5.6 nM	0.0095/min	· 169
BR3 tetraloop	3.7 nM	0.0475/min	1284

FIGURE 5

FIGURE 6

6A

6B